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Late production of CXCL8 in ruminant oro-nasal turbinate cells in response to *Chlamydia abortus* infection

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Abstract

Chlamydia abortus is an obligate intracellular bacterium that is an important cause of ovine abortion worldwide. There are reports of abortions in cattle, but these are very rare compared to the reported incidence in sheep. The bacterium is transmitted oro-nasally and can establish a sub-clinical infection until pregnancy, when it can invade the placenta and induce an inflammatory cascade leading to placentitis and abortion. Early host-pathogen interactions could explain differential pathogenesis and subsequent disease outcome in ruminant species. In this study, we assessed the ability of sheep and cattle oro-nasal turbinate cells to sense and respond to *C. abortus* infection. The cells expressed toll like receptor (TLR) 2, TLR4, nucleotide oligomerization domain (NOD) 1 and NOD-like receptor pyrin domain containing 3 (NLRP3) mRNA. In response to *C. abortus* infection, both ovine and bovine turbinate cells produce CXCL8 mRNA and protein, late in the bacterial developmental cycle, but do not produce IL-1 β or TNF- α . The UV-inactivated bacteria did not elicit a CXCL8 response, suggesting that intracellular multiplication of the bacteria is important for activating the signaling pathways. The production of innate immune cytokines from cattle and sheep turbinate cells in response to *C. abortus* infection was found to be largely similar.

Keywords

Chlamydia abortus; Innate immunity; Pattern recognition receptors; Inflammation; Ruminant

1 Introduction

Chlamydia abortus is a major infectious cause of ovine abortion worldwide (excluding Australia and New Zealand). The bacteria can also cause abortion in goats, but interestingly, is rarely linked to abortion in cattle even though cattle are susceptible to infection (Animal and Plant Health Agency (APHA), 2014; Longbottom and Coulter, 2003). *C. abortus* is transmitted oro-nasally and can infect the ewe at any time, persisting sub-clinically (site unknown) until late stage gestation when abortion facilitates the shedding of the bacteria for propagation of the infection cycle. Once a ewe has aborted due to *C. abortus* it does not abort again with re-infection indicative of the development of protective immunity (Rocchi et al., 2009).

During late-stage gestation, *C. abortus* recrudesces from its persistent state and invades the placenta inducing an inflammatory cascade typified by the infiltration of immune cells and production of TNF- α and CXCL8 (Kerr et al., 2005; Wheelhouse et al., 2009). To date, the strongest known single immunological correlate of protection to *C. abortus* is the production of IFN- γ ; however the ovine cellular source of IFN- γ and the innate immune mediators involved have yet to be elucidated (Entrican et al., 2010). Generally, the current understanding of innate and adaptive immune responses in small ruminants is relatively poor due to lack of specific immunological tools (Entrican and Lunney, 2012). The study of *C. abortus* in both small and large ruminants should therefore inform on pathogen-host co-evolution, thereby highlighting potentially important differences in immune recognition which contribute to pathological outcomes.

The genus *Chlamydia* currently comprises 12 species (Sachse et al., 2014; Vorimore et al., 2013) that share a characteristic biphasic developmental cycle, which involves extracellular and intracellular forms. The infectious elementary body (EB) is internalized by the host cell and induces the formation of an intracellular vacuole that allows transformation into the metabolically active reticulate bodies (RB), which multiply by binary fission (Abdelrahman and Belland, 2005). Consequently, both intracellular and extracellular pathogen recognition receptors (PRRs) are likely to be important for the detection of the bacteria by the host cell. A number of studies in mice and humans have highlighted the importance of TLR2 and TLR4 found on the host cell surface and intracellular NLRP3 and NOD1 for the activation of signaling pathways and release of pro-

inflammatory chemokines and cytokines in response to chlamydial infection (Rusconi and Greub, 2011; Shimada et al., 2012). In particular, CXCL8, TNF- α and IL-1 β have been identified as important innate immune cytokines produced in response to chlamydial infection (Rusconi and Greub, 2011).

Turbinate cells are components of the oral-nasal mucosa, which forms one of the first barriers of immune defense to pathogens. Therefore, the role of cattle and sheep turbinate cells to detect and activate the innate immune response to *C. abortus* infection was investigated. The expression profiles of pathogen recognition receptors (PRRs) by ovine and bovine turbinate cells and their production of innate immune chemokines and cytokines in response to *C. abortus* infection was assessed.

2 Materials and methods

2.1. Propagation of *C. abortus* stocks

C. abortus strain S26/3 was isolated at the Moredun Research Institute (MRI) and grown and titrated in HeLa cells, as previously described (Graham et al., 1995). Harvested bacteria were re-suspended in sucrose-phosphate-glutamate (SPG) (sucrose based medium containing FBS, antibiotics and fungicides) for storage at -80°C.

2.2. Cell culture and infection

Ruminant turbinate cells were generated at post-mortem by harvesting foetal turbinate tissues, which were then trypsinised to make a single cell suspension and cultured as previously reported (McClurkin et al., 1974) in order to create frozen cell banks. The primary cell lines used in this study represent different passages of cattle and sheep turbinate cells that were initially derived from the same fetal bovine or fetal ovine sources, respectively. These cells were screened and shown to be negative for pestivirus. After resuscitation, cells were adjusted to 1×10^5 /ml and grown to sub-confluence by adding 1ml/well in 24 well plates (Corning Costar, High Wycombe, UK) or 500ul/well in 8-well chamber slides (Nunc, Roskilde, Denmark). The culture medium consisted of Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Paisley, UK) supplemented with 10% heat inactivated fetal calf serum (PAA, Hanniger, Austria). As for the cell lines, the fetal calf serum was tested for live pestivirus and shown to be negative. The cells were then challenged with live *C. abortus* at a multiplicity of infection (MOI) of 10, with UV inactivated *C. abortus* (equivalent to MOI 10) and medium only.

UV treatment was shown to be effective at killing the bacteria as UV-inactivated *C. abortus* failed to form inclusions in HeLa cells (data not shown). Supernatants and lysates were harvested at 24, 48 and 72 hours from separate wells. The experiments were repeated on three separate occasions.

2.3. Immunofluorescence

Slides were fixed in cold 90% acetone 10% methanol and stored at -20°C. Slides were rehydrated in PBS prior to the addition of Image-iT® FX signal enhancer (Life Technologies) for 30 minutes in a humidity chamber protected from light. The slides were washed and then incubated with 1:1000 anti-*C. abortus* MOMP mouse monoclonal antibody (mAb) (4/11) (Santa Cruz Biotechnology, Heidelberg, Germany) for 30 minutes. After washing the slides three times, the Alexafluor 488 goat anti-mouse IgG (H+L) secondary polyclonal antibody (pAb) (Life Technologies) was added at 1:2000 for 30 minutes. The slides were washed three more times before mounting with ProLong Gold anti-fade reagent containing DAPI (Life Technologies).

2.4. Total RNA extraction and cDNA preparation

Cell lysates were harvested and stored in RLT lysis buffer at -80°C before total RNA was extracted using the RNeasy plus mini kit (Qiagen, Crawley, UK), following the manufacturer's instructions. The concentration of RNA was determined using a Nanodrop spectrophotometer (ThermoFisher Scientific, Rochester, NY, USA) and 1µg reverse transcribed into cDNA using Taqman® Reverse Transcription Reagents (Life Technologies). Reverse transcriptase was omitted from the process for the production of negative controls. Peripheral blood mononuclear cells (PBMC) were stimulated with Concanavalin A (Con A; ICN Biochemical, Cleveland, OH, USA) at 5µg/ml for 24 hours to generate cDNA to be used as positive controls.

2.5. Standard PCR

Primers were designed using primer 3 (<http://primer3.sourceforge.net/>) and details are shown in Table 1. PCR cycling conditions were 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, the appropriate annealing temperature for 30 seconds and 72°C for 1 minute and then a final extension of 72°C for 5 minutes. The cycle was finished with 5mins at 72°C. PCR products were run along with a 100bp ladder (New England Biolabs, Hitchin, UK) on a 1% agarose gel containing Gel Red™ (Biotum, Hayward, CA, USA) and visualized using UV light.

2.6. Taqman RT-PCR

Taqman RT-PCR was run using custom designed primers and probes for CXCL8. The commercially available eukaryotic 18s rRNA endogenous control (VIC/TAMRATM, Life Technologies), identified as the most stably expressed compared with GAPDH and β -actin, was used as the reference gene. The bovine CXCL8, TNF- α and IL-1 β primers and FAM/TAMRA probes used were previously designed (Galvao et al., 2012; Leutenegger et al., 2000), as were the ovine IL-1 β and TNF- α primers and FAM/TAMRA probes (Budhia et al., 2006). The ovine CXCL8 forward primer (5'-CACTGCGAAAATTCAGAAATCATTGTTA-3'), reverse primer (5'-CTTCAAAAATGCCTGCACAACCTTC-3') and probe (FAM-CCAACGGAAAAGAGGTGTGCTTAGACCCC-TAMRA) were designed as part of this study using Primer Express (Life Technologies). Samples were analyzed in triplicate on an ABI prism 7000 machine (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions; 50°C for 2 minutes, 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds followed by 60°C for 1 minute. Gene expression was quantified using the comparative $2^{-\Delta\Delta CT}$ method. Cells cultured without any exogenous stimulation in medium alone were harvested at each time point and used as a calibrator for all analyses of the treatment groups at that time point.

2.7. Cytokine ELISAs

Cytokine sandwich ELISAs using commercial pairs of mouse anti-sheep mAbs and rabbit anti-sheep pAbs to CXCL8 and IL-1 β (Bio-Rad AbD Serotec, Kidlington, UK), or rabbit anti-bovine pAb and biotin conjugated pAb to TNF- α (Thermoscientific, Cramlington, UK) were run as previously described (Rothel et al., 1998). Recombinant ovine CXCL8 expressed in Chinese Hamster Ovary cells (Wattegedera et al., 2004), recombinant bovine IL-1 β (Bio-Rad AbD Serotec) and recombinant bovine TNF- α (Bio-Rad AbD Serotec) were used to generate standard curves. The ELISAs were cross-reactive between cattle and sheep (Caswell et al., 1998; Tahoun et al., 2015; Wheelhouse et al., 2009) and had the following sensitivities: CXCL8 15pg/ml; TNF- α 1.95ng/ml and IL-1 β 60pg/ml.

2.8. Statistical analysis

Data included the expression profiles of CXCL8 mRNA and protein, and comprised three different passages of cell lines for each species (bovine and ovine) to which each level of treatment group (3 levels: live *C. abortus*, UV inactivated *C. abortus* and medium only) was applied and measured at three time points (24, 48 and 72 hours). The data on mRNA were obtained by comparative $2^{-\Delta\Delta CT}$ method by calibrating the expression for

medium control from each treatment group for each time point. Both mRNA and protein data showed increased variability with increased mean; hence the data on mRNA and protein were transformed by the logarithmic (after adding a constant value of 1) and square root transformation, respectively. The main effect of species, treatment group and time as well as possible two and three-way interaction effects were tested by univariate analysis of variance separately for the expression data of CXCL8 mRNA and protein. The blocking structure included the following categorical variables: cell line and interactions of cell line by treatment group and cell line by time. The overall statistical significance of the interaction effect was assessed using the F -statistic. If the overall test was statistically significant ($P < 0.05$), we obtained two-sided probabilities of comparisons of means of treatment groups at each time point. Finally, these probabilities were adjusted using a False Discovery Rate (FDR) approach (Benjamini and Hochberg, 1995) to take into account the multiple comparisons of means so that the expected proportion of false positives among all positives (i.e. rejecting the null hypothesis) was less than 5% and we denoted the adjusted probabilities as P_f . Statistical analyses were performed using GenStat (GenStat version 16.2) and R (R Core Team, version 3.1).

3 Results and discussion

Previous *in vivo* studies in sheep have demonstrated that *C. abortus* can induce abortion if inoculated over the tonsillar crypts (Jones and Anderson, 1988) and mucosal nasopharyngeal lymphoid tissues prior to a subsequent pregnancy (Longbottom et al., 2013). This suggests that turbinate cells are an appropriate *in vitro* model for studying initial infection. Initially, the ability of ovine and bovine turbinate cells to sustain an active *C. abortus* infection was assessed. Challenge with live *C. abortus* resulted in the formation of large intracellular inclusions (Figure 1 A, D) at 72 hours post-infection. Inclusions were not visible after exposure to UV-inactivated *C. abortus* however, visible fluorescent specks, an indication of dead bacteria, were present (Figure 1 B, E) which were not detected in the non-exposed controls (Figure 1 C, F).

As turbinate cells are a component of the oro-nasal mucosa, they are likely to be important sentinels of the immune system. We observed that ruminant turbinate cells express NLRP3, NOD1, TLR2 and TLR4 (Figure 2); thus ruminant turbinate cells fit the sentinel role as a varied complement of PRRs would be beneficial in the detection of pathogens. The human nasal mucosa expresses TLR2, TLR4 (Vandermeer et al., 2004), NOD1 and

NLRP3 (Bogefors et al., 2010), and this is the first study to demonstrate that ruminant turbinate cells also express these receptors. As these cells express a range of PRRs, they have the potential to respond to both intracellular and extracellular stages of *C. abortus* infection and activate signaling pathways leading to the transcription of cytokines and chemokines.

The expression profiles of CXCL8, TNF- α and IL-1 β mRNA and protein in three treatment groups (live *C. abortus*, UV-inactivated *C. abortus* and medium only) were assessed at three time points (24, 48 and 72 h). There was no IL-1 β mRNA or protein detected at any time points nor any TNF- α protein. Although low mRNA signal was detected in the TNF- α RT-PCR, it was around the threshold limit of detection for the assay. The absence of IL-1 β and TNF- α mRNA expression or protein production from ruminant turbinate cells is perhaps not too surprising as these cytokines are more commonly (but not exclusively) produced by myeloid cells, as has been shown for *C. trachomatis* and *C. pneumoniae* infections (Rusconi and Greub, 2011).

For the expression profile of CXCL8 mRNA, the interaction effect of treatment group and time was statistically significant ($P=0.006$). The mean CXCL8 mRNA expression, relative to the medium only group, in ovine and bovine turbinate cells for the live *C. abortus* group was significantly higher compared with the UV-inactivated *C. abortus* group at 72 hours post infection ($P_i<0.001$) (Figure 3A). The CXCL8 protein production mirrored the CXCL8 mRNA profiles: the interaction effect of treatment group and time was statistically significant ($P<0.001$). The live *C. abortus* treatment group produced significantly higher mean CXCL8 protein compared with the UV-inactivated *C. abortus* treatment group at 72 hours post infection in both ovine ($P_i<0.001$) and bovine ($P_i=0.028$) turbinate cells (Figure 3B). The live *C. abortus* group also showed higher mean CXCL8 protein production compared with the medium only group at 72 hours post infection in both ovine ($P_i<0.001$) and bovine ($P_i=0.022$) turbinate cells.

CXCL8 is a pro-inflammatory chemokine which functions as a chemotactic factor driving the recruitment of immune cells including neutrophils. Neutrophils have been identified as potential carriers of *C. pneumoniae*, which may perpetuate the survival and dissemination of the bacteria (Rupp et al., 2009; Zandbergen G. et al., 2004). Therefore, the production of CXCL8 by ruminant turbinate cells may aid in the dissemination of *C. abortus* from the site of infection. The increased CXCL8 production, however, occurred only at 72 hours, which is late in the chlamydial developmental cycle (Longbottom and Coulter, 2003). Earlier

studies identified that the late production of CXCL8 is a feature of *C. trachomatis* infection of epithelial cells (Buchholz and Stephens, 2006; Rasmussen et al., 1997). Potential explanations on CXCL8 production at a later stage include: inhibition of production by *C. abortus*, the pathogen associated molecular pattern (PAMP) threshold is not reached until the bacteria has multiplied or cell lysis is necessary for its production (Buchholz and Stephens, 2008; O'Connell et al., 2006).

We did not find any statistical evidence of interaction effects between species and treatment group, and between species and time for the expression of mRNA ($P>0.12$) and protein ($P>0.77$). These observations suggest that the production of CXCL8 from bovine and ovine turbinate cells on average had similar trends across treatment groups and time points. We also did not observe any evidence ($P_i>0.46$) that the mean CXCL8 protein production differed between the medium only group and UV-inactivated *C. abortus* challenged group at any time point in either species (Figure 3). This implies that intracellular detection of the active infection is necessary for the stimulation of CXCL8 mRNA expression. A previous study has also shown that CXCL8 production in ovine trophoblasts depends on challenge with live *C. abortus* compared to UV-inactivated *C. abortus* (Wheelhouse et al., 2009). Generally, pathogens are recognized by a range of PRRs which initiate a signal transduction cascade inducing activation of NF- κ B and MAPK kinases and production of pro-inflammatory chemokines and cytokines, including CXCL8 (Arthur and Ley, 2013). Previous studies have identified that the MAPK and NF- κ B signaling pathways regulated the induction of CXCL8 production following chlamydial infection (Buchholz and Stephens, 2006; Buchholz and Stephens, 2008; Rasmussen et al., 1997; Welter-Stahl et al., 2006). TLR2, which co-localises intracellularly with the inclusion, activates CXCL8 production from epithelial cells challenged with *C. trachomatis* (O'Connell et al., 2006). Intracellular NOD 1 mediates production of CXCL8 in an epithelial cell line challenged with *C. trachomatis* (Buchholz and Stephens, 2008). Our results showed that ruminant turbinate cells express TLR2, TLR4 and NOD1 (Figure 2), which indicates that *C. abortus* may activate similar signaling pathways for the production of CXCL8 within turbinate cells.

Understanding how the early innate immune response activates a protective adaptive immune response is critical for understanding disease pathogenesis and for the delivery of new vaccines. In this study, we observed that *C. abortus* is capable of infecting oro-nasal turbinate cells from cattle and sheep. Both ovine and bovine cells responded similarly to *C. abortus* infection by production of CXCL8 late in the chlamydial

216 developmental cycle with undetectable levels of IL-1 β and TNF- α . This suggests that innate cytokine and
217 chemokine production by turbinate cells is not a discriminating factor for disease pathogenesis between cattle
218 and sheep.

219 **Conflicts of interest:**

220 BioRad AbD Serotec distributes and markets ruminant immunological reagents produced by The Moredun
221 Research Institute and by The Roslin Institute at the University of Edinburgh. Both organizations receive
222 royalties from Bio-Rad AbD Serotec from the sale of these ruminant immunological reagents.

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232

233 Table 1: Standard PCR primer sequences for the detection of PRRs in ruminant turbinates cells

Target	Accession number	Forward Primer (5'-3')	Reverse Primer (5'-3')	Expected product size (base pairs)	Annealing temperature
Ovine NOD1	XM_004007930.1	CCA TCT TTG AAG TCG GGA TG	CGG GCT TTA TCA AGT TTC CA	378	50°C
Ovine NLRP3	XM_004008740.1	AAT CCC TTT CCC TGA GGT TG	GGG AAT GGT TGG TGC TTA GA	531	52°C
Ovine TLR2	NM_001048231.1	GCT CCT GTG ACT TCC TGT CC	AGT GGG AGA AGT CCA GCT CA	555	52°C
Ovine TLR4	NM_001135930.1	GGA CAA CCA ACC TGA AGC AT	TGA GGT TCC TTG GCA AAT TC	525	50°C
Bovine NOD1	NM_001256563.1	GCC TTC TTT GCT GCC CTT TTT C	CCC CAC CTT TTC ACT CTG TGT C	395	52°C
Bovine NLRP3	NM_001102219.1	TCA AGC TGT CCA CCA GAA TG	CAG AAT TCA CCA ACC CCA GT	711	52°C
Bovine TLR2	NM_174197.2	GGG GAT GAC AGA GGA TGA GA	GGC AGA AAG CTG ATG GAA AG	404	52°C
Bovine TLR4	NM_174198.6	GAC CCT TGC GTA CAG GTT GT	GGT TGA GTA GGG GCA TTT GA	538	52°C

234

235

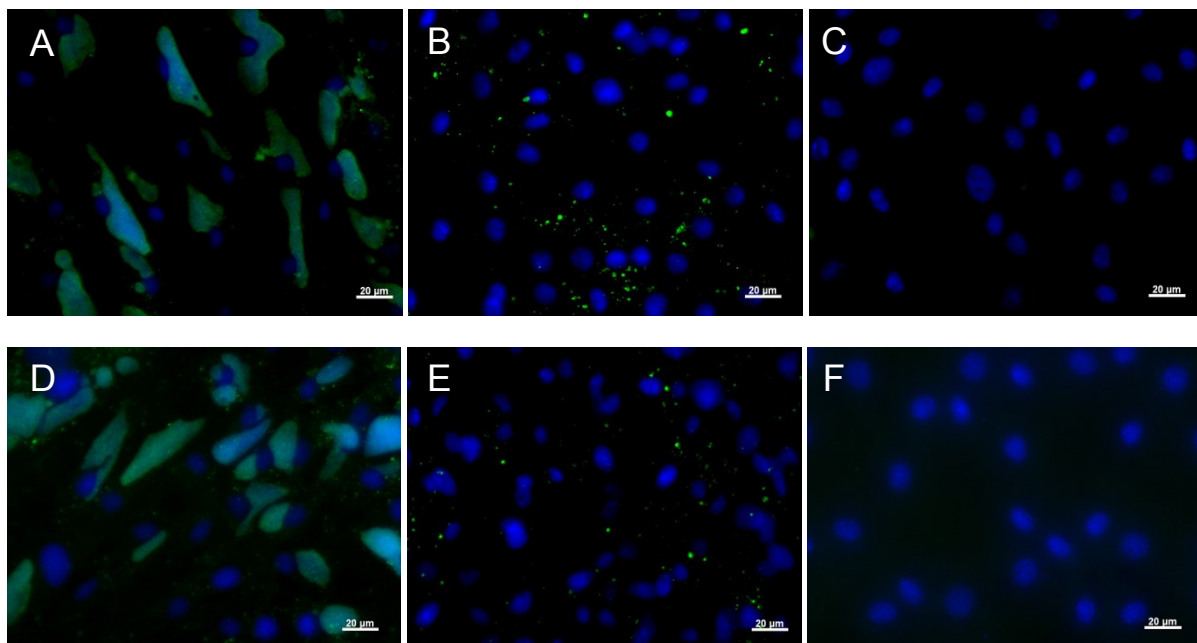


Figure 1: Immunofluorescent images of bovine (A-C) and ovine (D-F) turbinate cells demonstrating susceptibility to *C. abortus* infection.

C. abortus was labeled green with anti-*C. abortus* MOMP mouse monoclonal antibody 4/11 and visualized using Alexafluor 488 goat anti-mouse IgG secondary antibody and host cell nuclei are stained blue with DAPI. Turbinate cells challenged with MOI 10 live *C. abortus* (A, D), UV inactivated *C. abortus* (MOI 10 equivalent) (B, E) and medium only (C, F) for 72 hours. The scale bars correspond to 20μm.

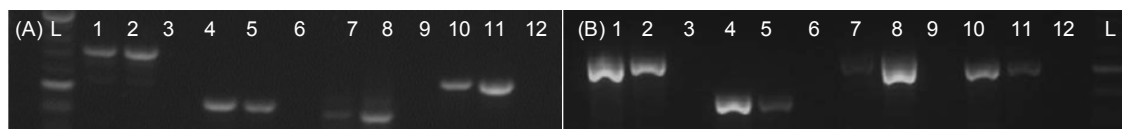
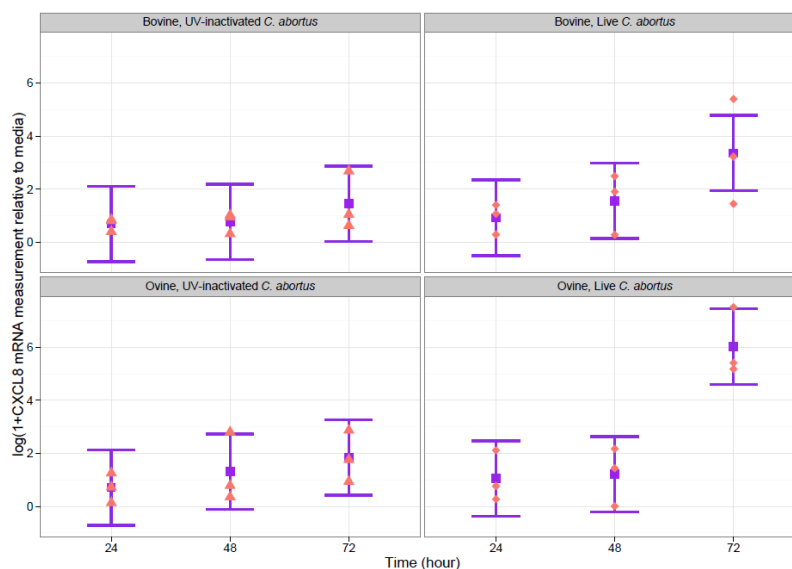


Figure 2: Expression of NLRP3, NOD1, TLR2 and TLR4 by bovine (A) and ovine (B) turbinate cells.

Total RNA was extracted from ruminant turbinate cells and Con A-stimulated PBMC, which was then reverse transcribed and PRRs amplified with specific primers (Table 1). Lanes 1, 4, 7 and 10: turbinate cells; Lanes 2, 5, 8 and 11: PBMC; Lanes 3, 6, 9 and 12: no reverse transcriptase control. Lanes 1-3: NLRP3; Lanes 4-6: NOD1; Lanes 7-9: TLR2; Lanes 10-12: TLR4. L: 100bp ladder. Products obtained were of the expected molecular weight.

A



B

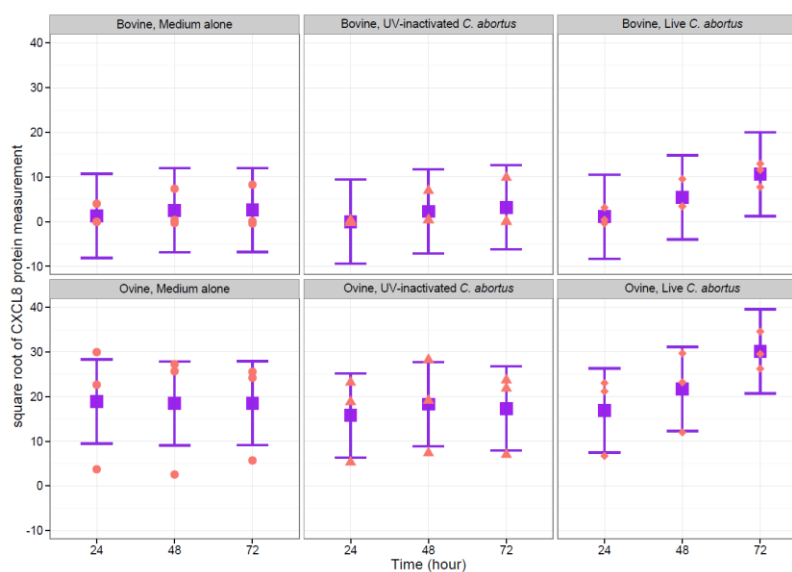


Figure 3: CXCL8 mRNA and protein production by ruminant turbinate cells in response to *C. abortus*.

The expression of (A) CXCL8 mRNA (on the logarithmic scale after adding a constant of one), measured in two treatment groups (response to UV-inactivated *C. abortus* and live *C. abortus*) relative to medium alone and (B) protein (on the square root scale) measured in three treatment groups (response to medium alone, UV-inactivated *C. abortus* and live *C. abortus*) at three different time points (24, 48 and 72 h) in two ruminant (bovine and ovine) turbinate cells. The plots show the mean expression (solid square) of mRNA and protein along with corresponding 95% confidence intervals (error bar). The plots also present the observed mRNA or protein data on each treatment group; with shapes (circle, triangle and diamond) representing each of the three treatment groups. The data on the expression profiles of mRNA and protein were separately analyzed using an analysis of variance approach incorporating main and interaction effects of species, treatment group and time and appropriate blocking structure.

Reference List

- Abdelrahman, Y.M., Belland, R.J., 2005. The chlamydial developmental cycle. *FEMS Microbiol Rev.* 29, 949-959.
- Animal and Plant Health Agency (AHPA), 1-10-2014. Veterinary Investigation Diagnosis Analysis (VIDA) report, 2013. <https://www.gov.uk/government/statistics/veterinary-investigation-diagnosis-analysis-vida-report-2013>. Last Accessed 13/05/14.
- Arthur, J.S., Ley, S.C., 2013. Mitogen-activated protein kinases in innate immunity. *Nat. Rev. Immunol* 13, 679-692.
- Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, 289-300.
- Bogefors, J., Rydberg, C., Uddman, R., Fransson, M., Mansson, A., Benson, M., Adner, M., Cardell, L.O., 2010. Nod1, Nod2 and Nalp3 receptors, new potential targets in treatment of allergic rhinitis? *Allergy* 65, 1222-1226.
- Buchholz, K.R., Stephens, R.S., 2006. Activation of the host cell proinflammatory interleukin-8 response by *Chlamydia trachomatis*. *Cell Microbiol* 8, 1768-1779.
- Buchholz, K.R., Stephens, R.S., 2008. The cytosolic pattern recognition receptor NOD1 induces inflammatory interleukin-8 during *Chlamydia trachomatis* infection. *Infect. Immun.* 76, 3150-3155.
- Budhia, S., Haring, L.F., McConnell, I., Blacklaws, B.A., 2006. Quantitation of ovine cytokine mRNA by real-time RT-PCR. *J Immunol Methods* 309, 160-172.
- Caswell, J.L., Middleton, D.M., Sorden, S.D., Gordon, J.R., 1998. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. *Vet Pathol* 35, 124-131.
- Entrican, G., Lunney, J.K., 2012. Veterinary Immunology Committee Toolkit Workshop 2010: progress and plans. *Vet Immunol Immunopathol* 148, 197-201.
- Entrican, G., Wattegedera, S., Wheelhouse, N., Allan, A., Rocchi, M., 2010. Immunological paradigms and the pathogenesis of ovine chlamydial abortion. *Am. J Reprod. Immunol* 64, 287-294.
- Galvao, K.N., Felipe, M.J., Brittin, S.B., Sper, R., Fraga, M., Galvao, J.S., Caixeta, L., Guard, C.L., Ricci, A., Gilbert, R.O., 2012. Evaluation of cytokine expression by blood monocytes of lactating Holstein cows with or without postpartum uterine disease. *Theriogenology* 77, 356-372.
- Graham, S.P., Jones, G.E., MacLean, M., Livingstone, M., Entrican, G., 1995. Recombinant ovine interferon gamma inhibits the multiplication of *Chlamydia psittaci* in ovine cells. *J Comp Pathol* 112, 185-195.
- Jones, G.E., Anderson, I.E., 1988. *Chlamydia psittaci*: is tonsillar tissue the portal of entry in ovine enzootic abortion? *Res Vet Sci* 44, 260-261.
- Kerr, K., Entrican, G., McKeever, D., Longbottom, D., 2005. Immunopathology of *Chlamydia abortus* infection in sheep and mice. *Res Vet Sci* 78, 1-7.
- Leutenegger, C.M., Alluwaimi, A.M., Smith, W.L., Perani, L., Cullor, J.S., 2000. Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan polymerase chain reaction. *Vet Immunol Immunopathol* 77, 275-287.
- Longbottom, D., Coulter, L.J., 2003. Animal chlamydioses and zoonotic implications. *J Comp Pathol* 128, 217-244.

- Longbottom, D., Livingstone, M., Maley, S., van der Zon, A., Rocchi, M., Wilson, K., Wheelhouse, N., Dagleish, M., Aitchison, K., Wattegedera, S., Nath, M., Entrican, G., Buxton, D., 2013. Intranasal infection with *Chlamydia abortus* induces dose-dependent latency and abortion in sheep. PLoS One 8, e57950.
- McClurkin, A.W., Pirtle, E.C., Coria, M.F., Smith, R.L., 1974. Comparison of low- and high-passage bovine turbinate cells for assay of bovine viral diarrhoea virus. Arch Gesamte Virusforsch. 45, 285-289.
- O'Connell, C.M., Ionova, I.A., Quayle, A.J., Visintin, A., Ingalls, R.R., 2006. Localization of TLR2 and MyD88 to *Chlamydia trachomatis* inclusions. Evidence for signaling by intracellular TLR2 during infection with an obligate intracellular pathogen. J Biol Chem. 281, 1652-1659.
- Rasmussen, S.J., Eckmann, L., Quayle, A.J., Shen, L., Zhang, Y.X., Anderson, D.J., Fierer, J., Stephens, R.S., Kagnoff, M.F., 1997. Secretion of proinflammatory cytokines by epithelial cells in response to Chlamydia infection suggests a central role for epithelial cells in chlamydial pathogenesis. J Clin. Invest 99, 77-87.
- Rocchi, M.S., Wattegedera, S., Meridiani, I., Entrican, G., 2009. Protective adaptive immunity to *Chlamydophila abortus* infection and control of ovine enzootic abortion (OEA). Vet Microbiol 135, 112-121.
- Rothel, J.S., Corner, L.A., Lightowlers, M.W., Seow, H.F., McWaters, P., Entrican, G., Wood, P.R., 1998. Antibody and cytokine responses in efferent lymph following vaccination with different adjuvants. Vet Immunol Immunopathol 63, 167-183.
- Rupp, J., Pfeleiderer, L., Jugert, C., Moeller, S., Klinger, M., Dalhoff, K., Solbach, W., Stenger, S., Laskay, T., van, Z.G., 2009. Chlamydia pneumoniae hides inside apoptotic neutrophils to silently infect and propagate in macrophages. PLoS One 4, e6020.
- Rusconi, B., Greub, G., 2011. Chlamydiales and the innate immune response: friend or foe? FEMS Immunol Med Microbiol 61, 231-244.
- Sachse, K., Laroucau, K., Riege, K., Wehner, S., Dilcher, M., Creasy, H.H., Weidmann, M., Myers, G., Vorimore, F., Vicari, N., Magnino, S., Liebler-Tenorio, E., Ruettger, A., Bavoil, P.M., Hufert, F.T., Rossello-Mora, R., Marz, M., 2014. Evidence for the existence of two new members of the family Chlamydiaceae and proposal of *Chlamydia avium* sp. nov. and *Chlamydia gallinacea* sp. nov. Syst Appl. Microbiol 37, 79-88.
- Shimada, K., Crother, T.R., Arditi, M., 2012. Innate immune responses to *Chlamydia pneumoniae* infection: role of TLRs, NLRs, and the inflammasome. Microbes Infect. 14, 1301-1307.
- Tahoun, A., Jensen, K., Corripio-Miyar, Y., McAteer, S.P., Corbishley, A., Mahajan, A., Brown, H., Frew, D., Aumeunier, A., Smith, D.G., McNeilly, T.N., Glass, E.J., Gally, D.L., 2015. Functional analysis of bovine TLR5 and association with IgA responses of cattle following systemic immunisation with H7 flagella. Vet Res 46, 9.
- Vandermeer, J., Sha, Q., Lane, A.P., Schleimer, R.P., 2004. Innate immunity of the sinonasal cavity: expression of messenger RNA for complement cascade components and toll-like receptors. Arch Otolaryngol. Head Neck Surg. 130, 1374-1380.
- Vorimore, F., Hsia, R.C., Huot-Creasy, H., Bastian, S., Deruyter, L., Passet, A., Sachse, K., Bavoil, P., Myers, G., Laroucau, K., 2013. Isolation of a New Chlamydia species from the Feral Sacred Ibis (*Threskiornis aethiopicus*): *Chlamydia ibidis*. PLoS One 8, e74823.
- Wattegedera, S., Sills, K., Howard, C.J., Hope, J.C., McInnes, C.J., Entrican, G., 2004. Variability in cytokine production and cell proliferation by mitogen-activated ovine peripheral blood mononuclear cells: modulation by interleukin (IL)-10 and IL-12. Vet Immunol Immunopathol 102, 67-76.
- Welter-Stahl, L., Ojcius, D.M., Viala, J., Girardin, S., Liu, W., Delarbre, C., Philpott, D., Kelly, K.A., Darville, T., 2006. Stimulation of the cytosolic receptor for peptidoglycan, Nod1, by infection with *Chlamydia trachomatis* or *Chlamydia muridarum*. Cell Microbiol 8, 1047-1057.

- 351 Wheelhouse, N., Wattegedera, S., Stanton, J., Maley, S., Watson, D., Jepson, C., Deane, D., Buxton, D.,
352 Longbottom, D., Baszler, T., Entrican, G., 2009. Ovine trophoblast is a primary source of TNFalpha during
353 *Chlamydomypha abortus* infection. J Reprod. Immunol 80, 49-56.
- 354 Zandbergen G., Gieffers, J., Kothe, H., Rupp, J., Bollinger, A., Aga, E., Klinger, M., Brade, H., Dalhoff, K., Maass,
355 M., Solbach, W., Laskay, T., 2004. *Chlamydia pneumoniae* multiply in neutrophil granulocytes and delay their
356 spontaneous apoptosis. J Immunol 172, 1768-1776.
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358